Dynamic changes in replication timing and gene expression during lineage specification of human pluripotent stem cells

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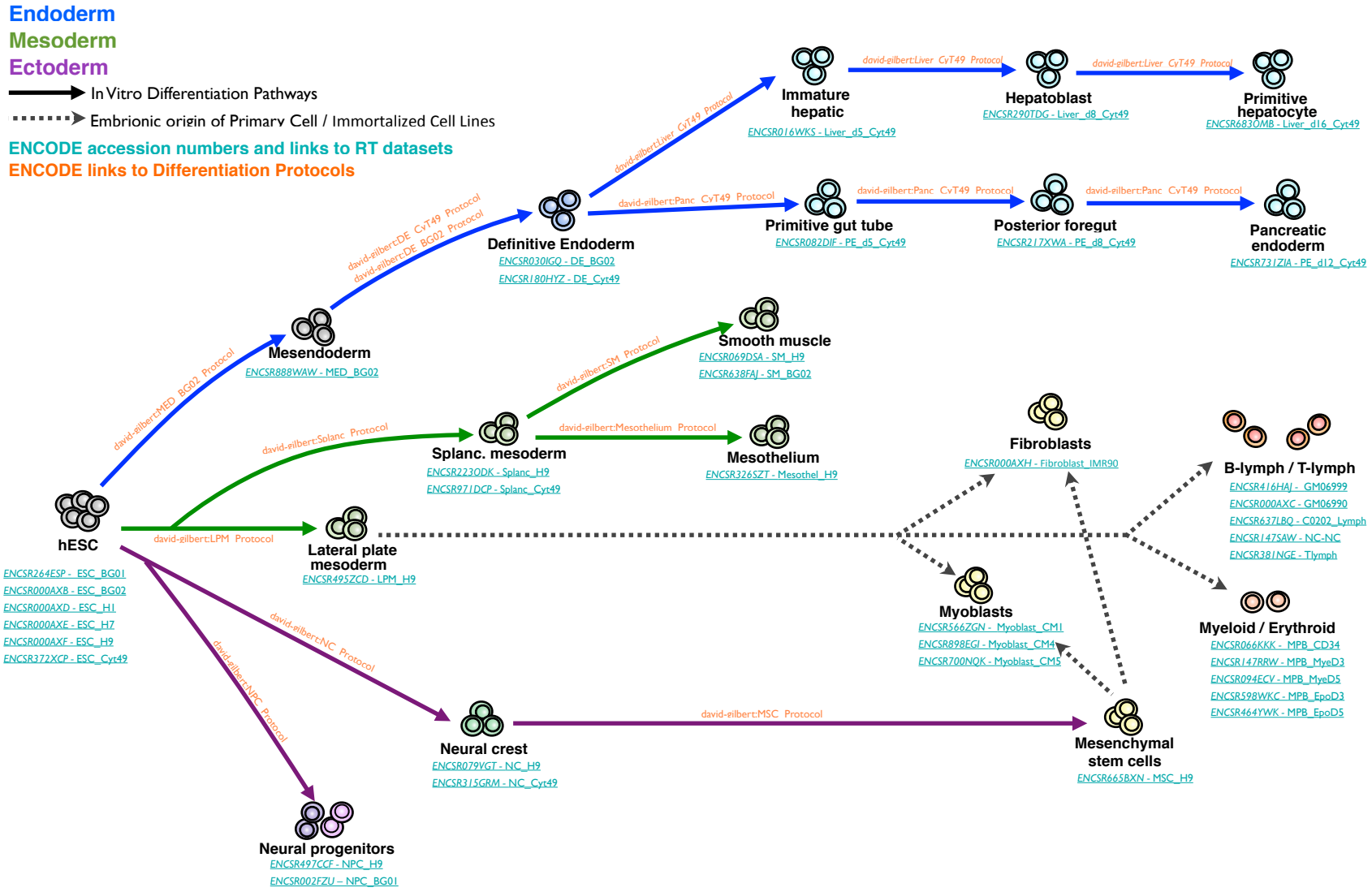
Supplementary methods

Replication Timing (RT) datasets of distinct human cell types



Supplementary differentiation protocols

1. Endoderm

Mesendoderm (MED), definitive endoderm (DE), Pancreatic (Panc) and hepatic (Liver) differentiation of hESC aggregates in suspension.

Pancreatic samples consisted of pancreatic endoderm and immature endocrine cells and were made using a 12-day differentiation regime (Figure 1) as described in detail in (Schulz et al. 2012). Briefly, CyT49 hESC were aggregated in rotational suspension culture, then differentiated in a four-step sequential process to mesendoderm (MIXL1$^+$), definitive endoderm (SOX17$^+$, FOXA2$^+$); primitive gut tube (HNF1B$^+$, HNF4A$^+$); posterior foregut (PDX1$^+$); and pancreatic endoderm (NKX6-1$^+$, PTF1A$^+$) and endocrine cells (NGN3$^+$, NKX2-2$^+$, PAX4$^+$, PAX6$^+$), according to the following conditions:

Time point	Stage	Media Condition	Rotation Speed
d(-1)	Aggregation	XF HA	95
d0	1	r0.2FBS-ITS1:5000 A100 W50	95
d1		r0.2FBS-ITS1:5000 A100	95
d2	2	r0.2FBS-ITS1:1000 K25 IV	95
d3		r0.2FBS-ITS1:1000 K25	95
d4		r0.2FBS-ITS1:1000 K25	105
d5	3	db-CTT3 N50	105
d6		db-CTT3 N50	105
d7		db-CTT3 N50	105
d8	4	db-N50 K50 E50	105
d9		db-N50 K50 E50	95
d10		db-N50 K50 E50	95
d11		db-N50 K50 E50	95
d12	harvest		

XF HA: DMEM/F12 containing GlutaMAX (Life Technologies, cat#10565-042), supplemented with 10% v/v of Xeno-free KnockOut Serum Replacement (Life Technologies, cat#12618-001), 1% v/v non-essential amino acids (Life Technologies, cat#11140-050), 1% v/v penicillin/streptomycin (Life Technologies, cat#15070-063), 10 ng/mL heregulin-1β (Peprotech, cat#100-03) and 10 ng/mL activin A (R&D Systems, cat#338-AC). **r0.2FBS**: RPMI 1640 (Mediatech, cat#15-040-CV), 0.2% FBS (HyClone, cat#SH30070.03), 1x GlutaMAX-1 (Life Technologies, cat#35050-061), 1% v/v penicillin/streptomycin. **ITS**: Insulin-Transferrin-Selenium (Life Technologies, cat#41400-045) diluted 1:5000 or 1:1000. **A100**: 100 ng/mL recombinant human Activin A (R&D Systems, cat#338-AC). **W50**: 50 ng/mL recombinant mouse Wnt3A (R&D Systems, cat#1324-WN). **K25**: 25 ng/mL recombinant human KGF (R&D Systems, cat#251-KG). **IV**: 2.5 mM TGF-β RI Kinase inhibitor IV (EMD Bioscience, cat#616454). **db**: DMEM HI Glucose (HyClone cat#SH30081.01) supplemented with 0.5x B-27 Supplement (Life Technologies, cat#17504-044), 1x GlutaMAX-1 and 1% v/v penicillin/streptomycin. **CTT3**: 0.25 mM KAAD-Cyclopamine (Toronto Research Chemicals, cat#K171000) and 3 nM TTNPB (Sigma-Aldrich, cat#T3757). **N50**: 50 ng/mL recombinant human Noggin (R&D Systems, cat#3344-NG). **K50**: 50 ng/mL recombinant human KGF (R&D Systems, cat#251-EG). **E50**: 50 ng/mL recombinant human EGF (R&D Systems, cat#236-EG).

Liver differentiation was carried out using modifications to stages 2-4 of the above protocol (Figure 1), resulting in an APF^+, $Albumin^+$ and TAT^+ cell population.

Time point	Stage	Media Condition	Rotation Speed
d(-1)	Aggregation	XF HA	95
d0	1	r0.2FBS-ITS1:5000 A100 W50	95
d1		r0.2FBS-ITS1:5000 A100	95
d2	2	r0.2FBS-ITS1:1000 B100 F25	95
d3		r0.2FBS-ITS1:1000 B100 F25	95
d4		r0.2FBS-ITS1:1000 B100 F25	105
d5	3	db-B100	105
d6		db-B100	105
d7		db-B100	105
d8	4	db-K50 E50	105
d9		db-K50 E50	95
d10		db-K50 E50	95
d11		db-K50 E50	95
d12		db-K50 E50	95
d13		db-K50 E50	95
d14		db-K50 E50	95
d15		db-K50 E50	95
d16	harvest		

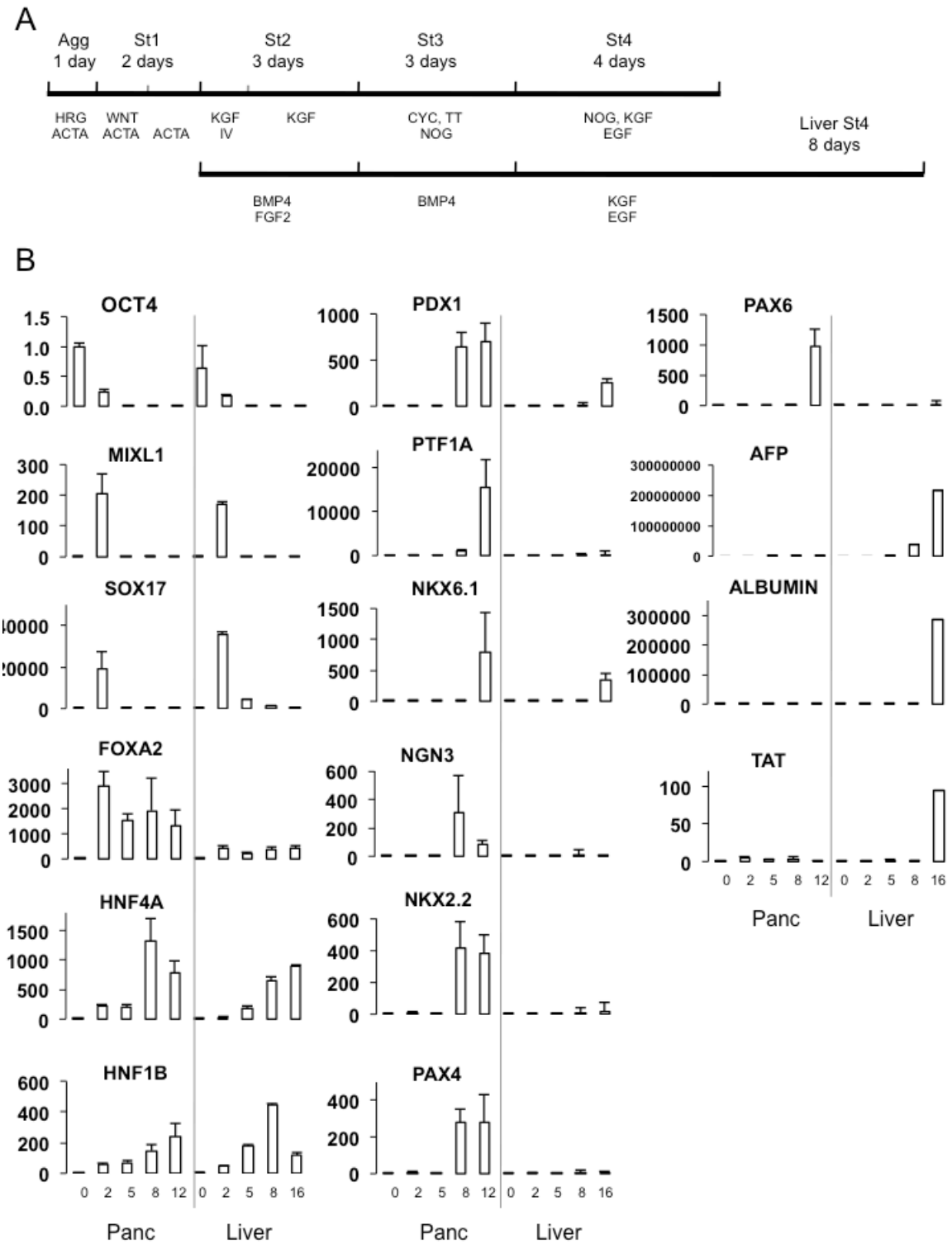
B100: 100 ng/mL BMP4 (R&D Systems, cat#314-BP)

F25: 25 ng/mL FGF2 (Sigma, cat#F0291)

FIGURE 1 (next page)

Directed pancreatic and liver differentiation of CyT49 in suspension culture. (A) Schematic timeline of pancreatic (upper) and liver differentiation (lower) of CyT49 cells. Aggregates of undifferentiated hESC were generated in rotational suspension culture, and then differentiated through a 4-step process. Growth factors and small molecule inhibitors used to specify differentiation are indicated. HRG, heregulin 1β; ACTA, Activin A; WNT, Wnt3A; KGF, keratinocyte growth factor (FGF7); IV, TGF- β RI kinase inhibitor IV; CYC, cyclopamine; TT, TTNBP (retinoic acid analog); NOG, noggin; EGF, epidermal growth factor. (B) The dynamics of gene expression (qPCR) demonstrated that undifferentiated cells (OCT4) were directed through mesendoderm (MIXL1), definitive endoderm (SOX17, FOXA2), primitive gut tube (HNF4A, HNF1B), posterior foregut (PDX1), to form pancreatic endoderm (NKX6-1, PTF1A) and endocrine cells (NGN3, NKX2-2, PAX4, PAX6). BMP4 and FGF2 driven specification in stages 2 and 3 resulted in the generation of hepatic lineages (AFP, Albumin, TAT). Panc: pancreatic differentiation conditions, Liver: liver differentiation conditions. Day of differentiation indicated on x-axis.

FIGURE 1


A
Agg
1 day
St1
2 days
St2
3 days
St3
3 days
St4
4 days
HRG
ACTA
WNT
ACTA
ACTA
KGF
IV
KGF
CYC, TT
NOG
NOG, KGF
EGF
Liver St4
8 days
BMP4
FGF2
BMP4
KGF
EGF
B
OCT4
PDX1
PAX6
MIXL1
PTF1A
AFP
SOX17
NKX6.1
ALBUMIN
FOXA2
NGN3
TAT
HNF4A
NKX2.2
HNF1B
PAX4
0 2 5 8 12 0 2 5 8 16
Panc
Liver
Panc
Liver
Panc
Liver

2. Mesoderm

Lateral plate mesoderm (LPM):

Lateral plate mesoderm cells (T+, Nanog-) were derived from hESC in DMEM/F12, with 100 ng/ml Activin A, 20 ng/ml Fgf2, 25 ng/ml Wnt3a (R&D Systems) and BMP4 (R&D Systems) at 100 ng/ml for 2 days (Figure 2).

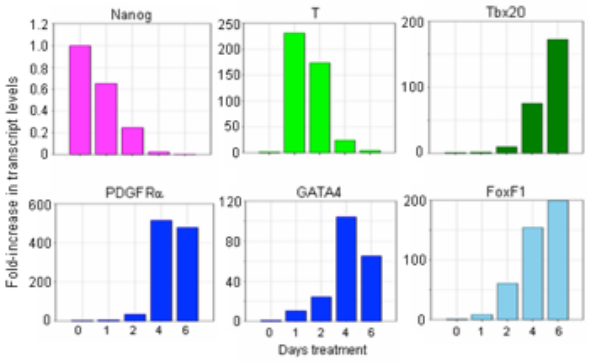


Figure 2. Gene expression profiling (qPCR) of hESCs differentiated towards a mesoderm fate in defined medium. LPM samples were collected at day 2 of differentiation.

Splanchnic mesoderm (Splanc):

Splanchnic mesoderm (PDGFRα+, GATA4+, FoxF1+, ISL1+ and Tbx20+) were derived from hESC in DMEM/F12, with 100 ng/ml Activin A, 20 ng/ml Fgf2, 25 ng/ml Wnt3a (R&D Systems) and BMP4 (R&D Systems) at 100 ng/ml for 4 days (Figure 3).

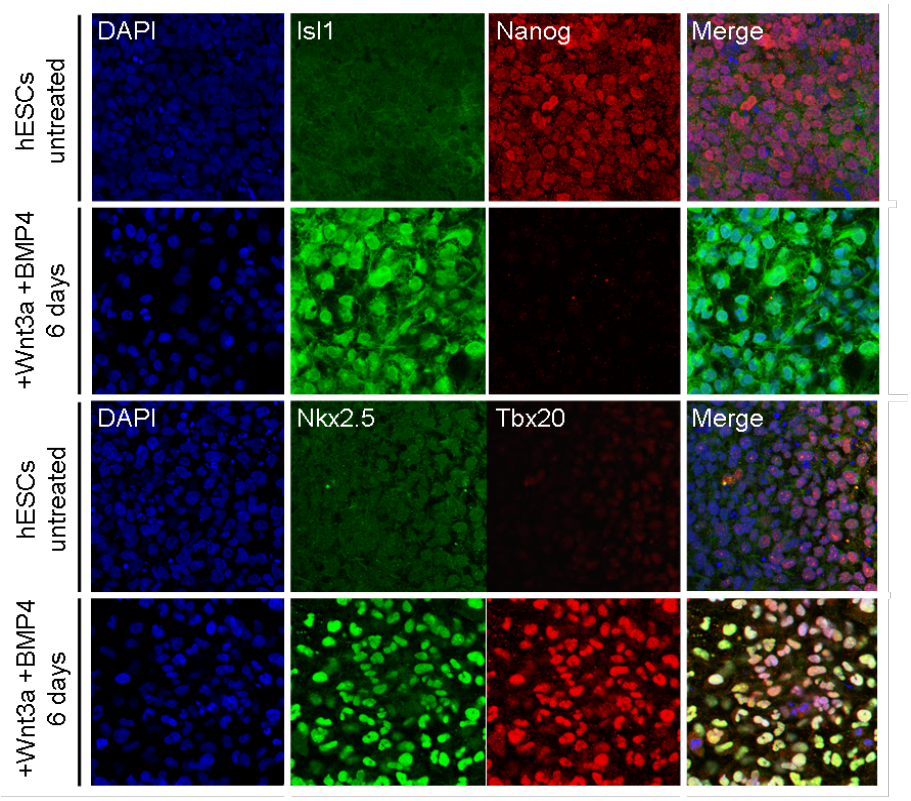


Figure 3. Generation of Isl1+ splanchnic mesoderm from hESCs. DAPI staining and immunofluorescence analysis for Isl1, Nanog, Nkx2.5 and Tbx20 of untreated (hESCs) and Wnt3a/BMP4 at day 4 of differentiation.

Mesothelium:

Mesothelial cells (Tbx5+, Raldh2+, WT1+) were derived from splanchnic mesoderm cells in the presence of Wnt3a (25ng/ml), BMP4 (50ng/ml) and all-trans retinoic acid (Sigma; 4μM) for 16 days (Figure 4).

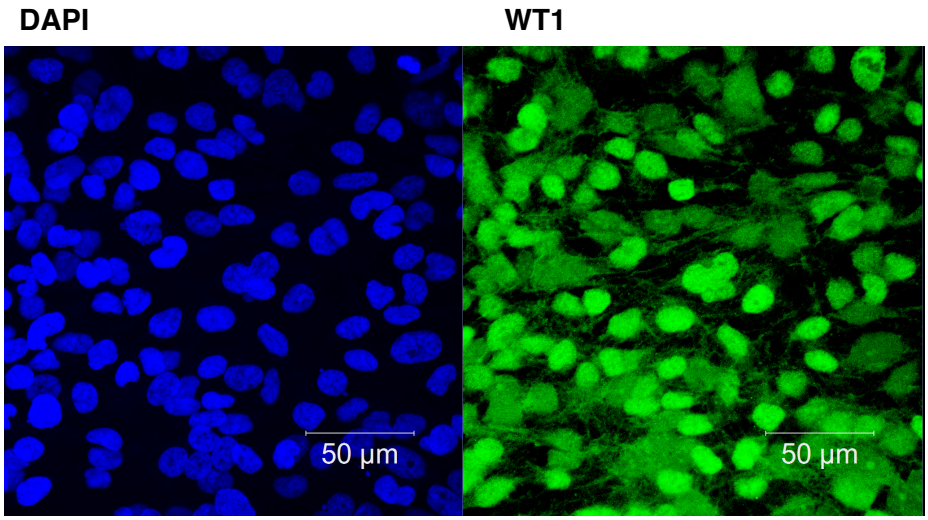


Figure 4. Immunofluorescence analysis of smooth muscle actin (SMA) and calponin for cells at day-16 of differentiation with Wnt3a/BMP4.

Smooth Muscle:

Smooth muscle cells (SMA+, SM-MHC+, CALD1+, calponin+) were differentiated from hESC in DMEM/F12, with 100 ng/ml Activin A, 20 ng/ml Fgf2, 25 ng/ml Wnt3a and BMP4 at 100 ng/ml for 18 days (Figure 5).

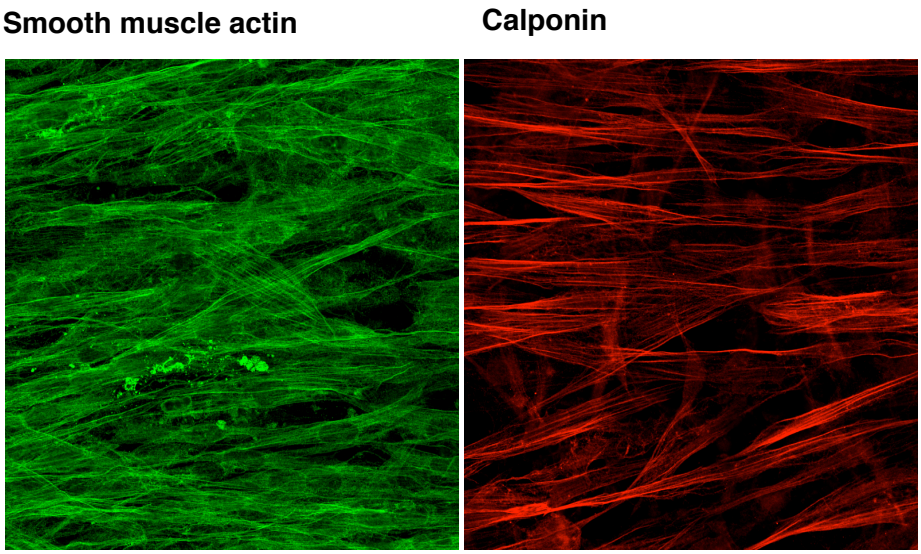


Figure 5. Immunofluorescence analysis of smooth muscle actin (SMA) and calponin for cells at day-16 of differentiation with Wnt3a/BMP4.

3. Neural crest (NC) and mesenchymal stem cells (MSC) differentiation.

For direct neural crest differentiation, hES cells were plated at a density of 1 × 10^5 cells/cm2 in defined media lacking Activin A and supplemented with 2 micromolar GSK3 inhibitor IX (BIO) and 20 micromolar SB 431542 (Menendez et al. 2011; 2013). Media was replaced every day. Neural crest identity was confirmed by immunocytochemistry (Figure 6) and RT-PCR positive detection of p75, Hnk1, AP2 and FoxD3 NC markers, as well as negative detection of hESCs markers (Sox2, Oct4, Nanog) and neural progenitor markers (Pax6, Sox2). For mesenchymal differentiation, neural crest cells were cultured in media containing 10% FBS and passed every 4–5 d, MSCs were CD73+, CD44+ and CD13+ by day 5 (Figure 7).

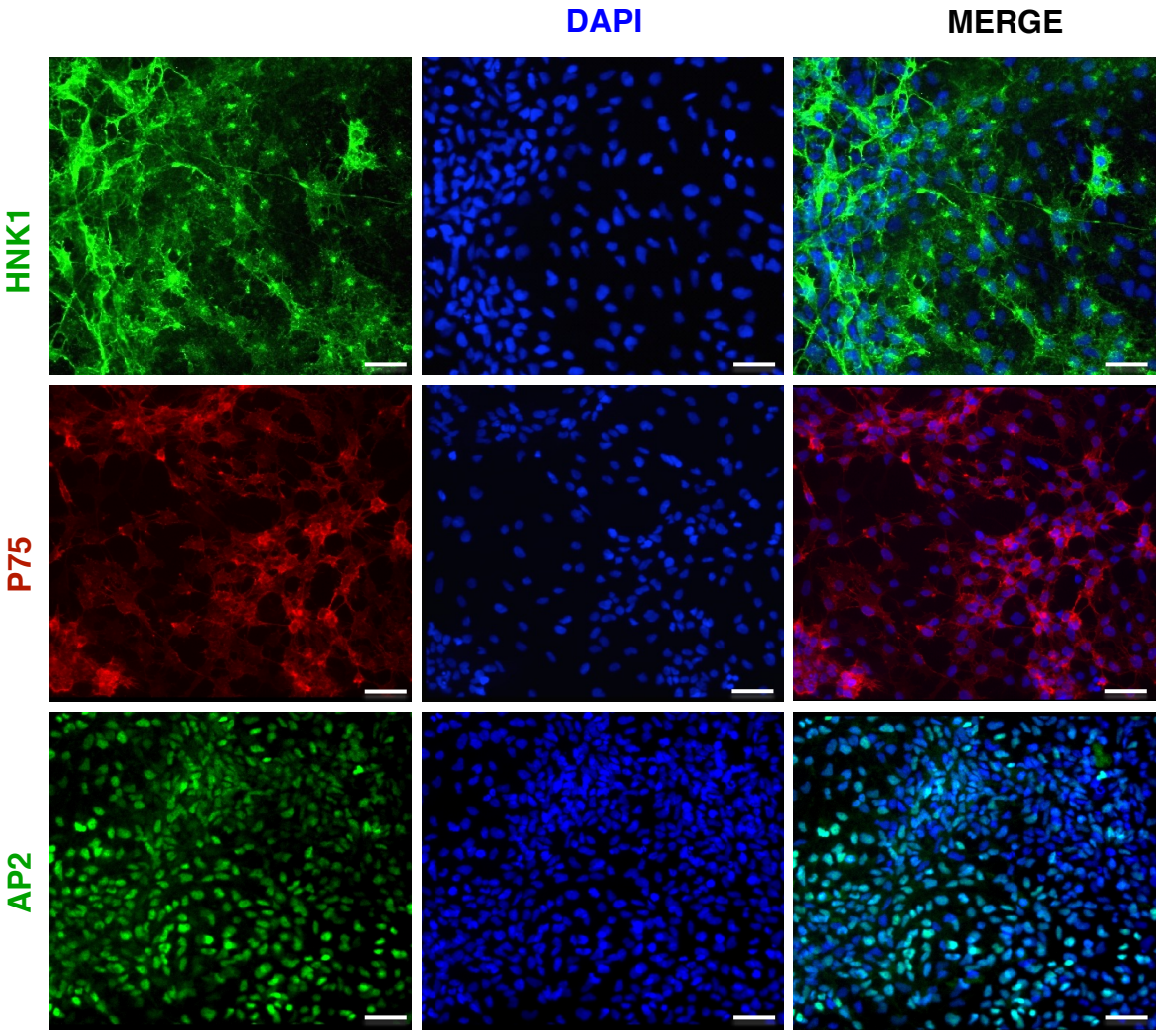


Figure 6. Immunofluorescence analysis for Hnk1, p75 and AP2 for hES cells differentiated with ActivinA and GSK3 inhibitor IX (BIO).

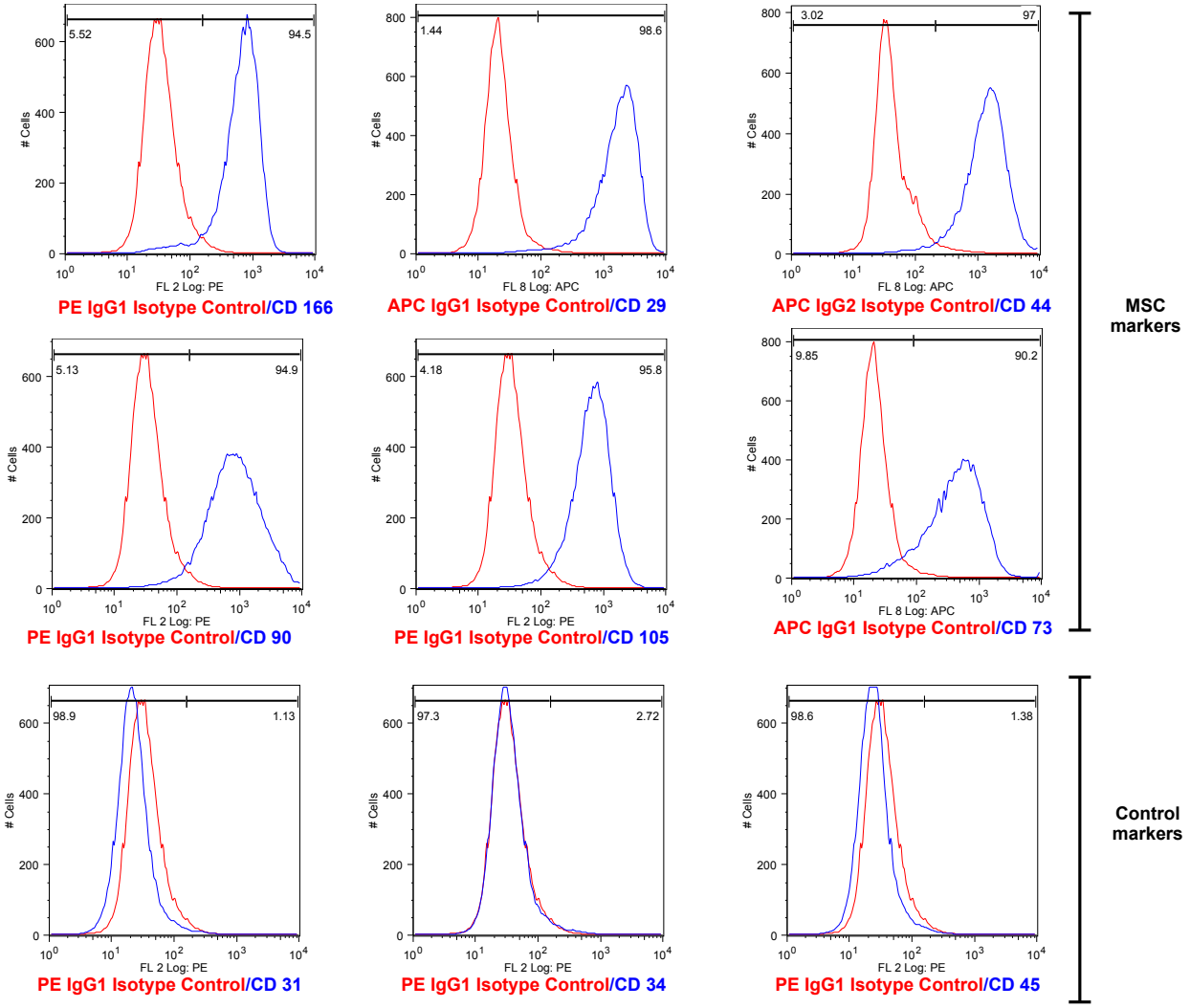


Figure 7. Flow cytometry analysis of differentiated cells with ActivinA and GSK3 inhibitor IX (BIO) after addition of FBS using distinct mesenchymal stem cells markers (CD166, CD29, CD44, CD73, Cd90, Cd105).

4. Ectoderm: Neural progenitors differentiation.

Undifferentiated hESCs were plated and expanded in DM containing Noggin and SB431542 to induced neural differentiation. Cultures were split at day 7 and 14. FGF2 was added at day 14 to promote neural progenitor expansion. On day 28 the culture was split and neural progenitors expanded in DMEM/F12/B27 containing heregulin and FGF2. Q-PCR analysis demonstrated that these NP cells expressed SOX1, PAX6 (Figure 8), but were negative for OCT4, Rex1, Nanog, and CD9, in comparison to undifferentiated cells. Immunofluorescence confirmed that these progenitors were negative for OCT4, but expressed Sox2 and Nestin. This population can be expanded and maintained for >3 months in defined conditions (Schulz et al. 2003; 2004).

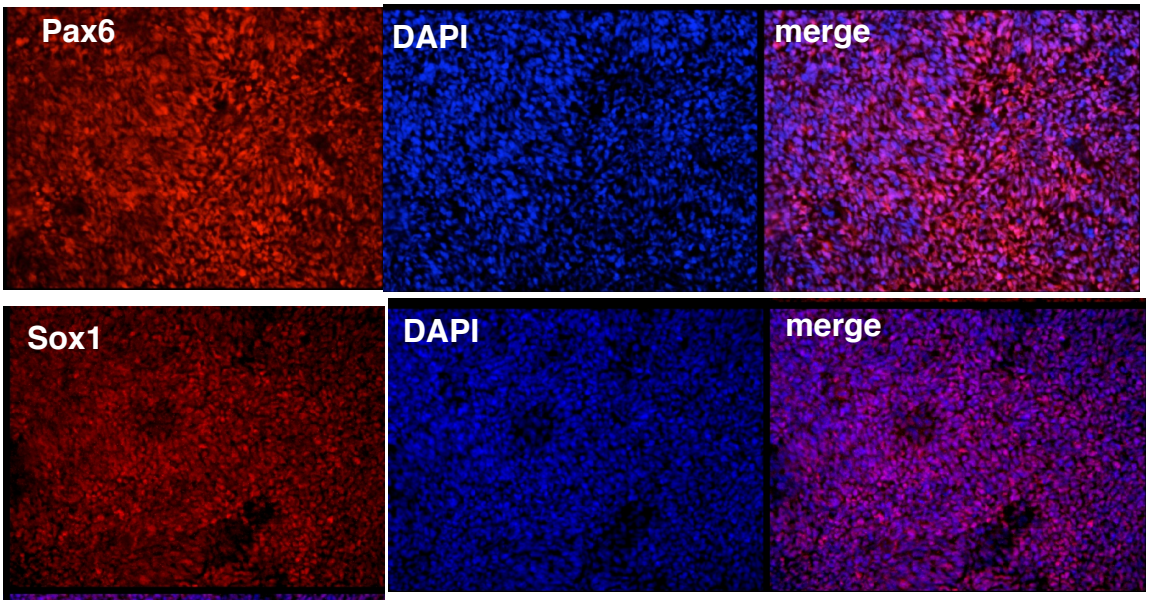


Figure 8. DAPI staining and immunofluorescence analysis of hESC derived NPCs for Pax6 and Sox1.

5. Myeloid and erythroid differentiation from CD34+ progenitors.

Human CD34+ peripheral blood stem and progenitor cells were used for cytokine-mediated erythroid or myeloid differentiation *in vitro* (Migliaccio et al. 2009; Mahajan et al. 2009; Heike and Nakahata 2002; Mayani et al. 1993; Manz et al. 2002). These cells, harvested from G-CSF-treated normal adults undergoing apheresis as stem cell transplant donors, were obtained from the Yale Center for Excellence in Molecular Hematology (Dr. Diane Krause, director) as de-identified samples. The CD34+ cells were selected using the Isolex 300i Magnetic Cell Selection System (Baxter), and cultured in StemSpan Expansion Medium (Stem Cell Technologies) with 1X Stem Span CC100 recombinant human cytokine cocktail containing 100 ng/mL Flt3 ligand, 100 ng/mL stem cell factor, 20 ng/mL IL-3, and 20 ng/mL IL-6. After seven days of expansion in culture, the cell number increased over 40 fold. The cells were differentiated by addition of lineage related cytokines

For myeloid differentiation, CD45RA+ cells were used and the stem cell expansion medium was supplemented as follows: Days 0-4: 50 ng/ml stem cell factor, 100 ng/ml Flt3 ligand, 5 ng/ml IL-3, 5 ng/ml GM-CSF, 30 ng/ml G-CSF; Days 4-7: 5 ng/ml IL-3, 30 ng/ml G-CSF; Days 7-12: 30 ng/ml G-CSF.

Erythroid differentiation was induced in the CD45RA- fraction of cells by an erythroid “cocktail” supplementing the stem cell expansion medium with 10ng/ml SCF, 5ng/ml IL3, recombinant erythropoietin 1 unit/ml, dexamethasone with a final concentration of 2 micromolar, and Estradiol with a final concentration of 0.2 micromolar.

Gene expression analysis

Total RNA was extracted from snap-frozen sample pellets (Ambion mirVana Kit, Life Technologies, Inc.) according to the manufacturer's protocol. RNA quantity (QubitTM RNA BR Assay Kits, Life Technologies, Inc.) and quality (RNA6000 Nano Kit and Bioanalyzer 2100, Agilent) was determined to be optimal for each sample prior to further processing. 200 ng RNA per sample was amplified using the Total PrepTM RNA Amplification Kit (Illumina, Inc.) according to manufacturer's protocol and quantified as above. 750 ng labeled RNA/sample was hybridized to humanHT-12v4 Expression BeadChips (Illumina, Inc.), scanned with an iScan (Illumina, Inc.). In GenomeStudio, probes were filtered for those detected at $p<0.01$ in at least 1 sample and exported for normalization in R using robust spline normalization (RSN). Probes in the array (47,000) were analyzed by BLAT (Kent 2002) and only unambiguous and highly specific probes were included in the analysis (25,000 specific probes against 16,000 RefSeq genes).

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